JC07 Rec'd PCT/PTO 01 FEB 2001

FORM PTO-1390 (Modified) (REV 11-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S APPLICATION NO. (IF KNOWN, SEE 37 CFR)
PENN-0749	09/762023

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US99/17386	2 August 1999	4 August 1998

TITLE OF INVENTION

Targeting and Prolonging Association of Drugs to the Luminal Surface of the Pulmonary Vascular Endothelial Cells

APPLICANT(S) FOR DO/EO/US

MUZYKANTOV, Vladmir R. et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). – Unexecuted
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

1) Courtesy copy of International Application
2) Executed Verified Statement Claiming Small Entity Status
3) Written Opinion and Response thereto
4) Return Post Card

"Express Mail" Label No. **EL722986054US**
Date of Deposit **February 1, 2001**

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231.

By Deborah Ehret
Typed Name: Deborah Ehret

JC17 Rec'd PCT/PTO 01 FEB 2001

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/762025	PCT/US99/17386	PENN-0749

21. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1,000.00**
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO	**$860.00**
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$710.00**
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$690.00**
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	8 - 20 =	0	x $18.00	**$0.00**	
Independent claims	2 - 3 =	0	x $80.00	**$0.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$860.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**. ☒				**$430.00**	
SUBTOTAL =				**$430.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$430.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$430.00**	
				Amount to be: refunded	$
				charged	$

☐ A check in the amount of to cover the above fees is enclosed.

X Credit Card Payment form in the amount of $430.00 for filing fee

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **50-1619** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Jane Massey Licata, Reg. No. 32,257
Kathleen A. Tyrrell, Reg. No. 38,350

Licata & Tyrrell P.C.
66 E. Main Street
Marlton, New Jersey 08053

Telephone: (856) 810-1515
Facsimile : (856) 810-1454

Kathleen A. Tyrrell
SIGNATURE

TYRRELL, Kathleen A.
NAME

38,350
REGISTRATION NUMBER

1 February 2001
DATE

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27 (d)) - NONPROFIT ORGANIZATION	Docket No. FENN-0749

Serial No.	Filing Date	Patent No.	Issue Date
Not Yet Assigned	Herewith		

Applicant/ Patentee: MUZYKANTOV, Vladimir R.

Invention:

Vascular Endothelial Cells

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: The Trustees of the University of Pennsylvania

ADDRESS OF ORGANIZATION: 3700 Market Street, Suite 300
Philadelphia, Pennsylvania

TYPE OF NONPROFIT ORGANIZATION:

- ☒ University or other Institute of Higher Education
- ☐ Tax Exempt under Internal Revenue Service Code (26 U.S.C. 501(a) and 501(c)(3))
- ☐ Nonprofit Scientific or Educational under Statute of State of The United States of America
  Name of State: Citation of Statute:
- ☐ Would Qualify as Tax Exempt under Internal Revenue Service Code (26 U.S.C. 501(a) and 501(c)(3)) if Located in The United States of America
- ☐ Would Qualify as Nonprofit Scientific or Educational under Statute of State of The United States of America if Located in The United States of America
  Name of State: Citation of Statute:

I hereby declare that the above-identified nonprofit organization qualifies as a nonprofit organization as defined in 37 C.F.R. 1.9(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in:

- ☒ the specification to be filed herewith.
- ☐ the application identified above.
- ☐ the patent identified above.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the above-identified nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed on the next page and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Copyright 1994 Legalsoft

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

- ☒ no such person, concern or organization exists.
- ☐ each such person, concern or organization is listed below.

FULL NAME ______________________
ADDRESS ______________________
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME ______________________
ADDRESS ______________________
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME ______________________
ADDRESS ______________________
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME ______________________
ADDRESS ______________________
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Alfred J. Glessner

TITLE IN ORGANIZATION: Director, Operators and Licensing

ADDRESS OF PERSON SIGNING: Center for Technology Transfer
3700 Market Street, Suite 300
Philadelphia, Pennsylvania

SIGNATURE: Alfred J. Glessner DATE: 25 JAN 01

09762467 . 012501

13 Rec'd PCT/PTO 01 FEB 2001
09/762023

TARGETING AND PROLONGING ASSOCIATION OF DRUGS TO THE LUMINAL SURFACE OF THE PULMONARY VASCULAR ENDOTHELIAL CELLS

Background of the Invention

Pulmonary vasculature is anatomically predisposed to deposition of fibrin and thromboemboli formed in the vasculature (for example, upon deep vein thrombosis). Both emboli and fibrin lodged in the lung play an important role in the pulmonary and cardiovascular pathology and contribute significantly to morbidity and mortality of disease conditions including, but not limited to, thrombosis, atherosclerosis, deep vein thrombosis, diabetes, adult respiratory distress syndrome, pulmonary embolism, shock and sepsis. Anticoagulants (e.g., heparin) are useful in preventing formation of intravascular fibrin clots, whereas fibrinolytics (e.g., plasminogen activators) are useful for dissolution of fibrin clots. Both anticoagulants and fibrinolytics, however, undergo inactivation and elimination from the bloodstream. This restricts their applicability for treatment of pulmonary embolism. Administration of large doses and/or multiple injections of a drug to compensate for elimination/inactivation impose inconvenience in treatment and high risk of harmful side effects. Uncontrolled bleeding is an example of such side effects of prolonged administration or a large dose of anticoagulants or fibrinolytics.

Augmentation of anticoagulant or/and fibrinolytic potential of the luminal surface of endothelial cells lining

pulmonary vessels thus represents an important therapeutic strategy for treatment or/and prevention of disease conditions associated with or manifested by pulmonary embolism and fibrin deposition. Because these therapeutics must have access to the blood components in order to control coagulation or activate fibrinolysis, a requirement for such a strategy is that the anticoagulant or fibrinolytic agent be associated for a prolonged time with the luminal surface of the pulmonary endothelium.

One approach to attain this objective is to conjugate a drug to an antibody against surface endothelial molecules. This conjugation provides selective delivery, also referred to herein as targeting, of a drug to endothelium and prolonged association of a drug with endothelium. Therapeutic enzymes and genetic material conjugated to such antibodies have been demonstrated to bind to the endothelial cells *in vitro* and *in vivo* after injection in animals. Since the lungs contain approximately 30% of the total amount of endothelial cells in the body and receive a whole cardiac output of venous blood, antibodies against endothelial antigens tend to accumulate in the lung after intravenous injection. For example, Kennel et al. have described an antibody against thrombomodulin which recognizes endothelial surface *in vivo*, accumulates in the pulmonary vasculature and is capable of delivery of conjugated liposomes to the pulmonary endothelium (Kennel et al. 1990 *Nucl. Med. Biol.* 17:193-100; Trubetskoy et al. 1992 *Biochim. Biophys. Acta* 1131:311-313). An antibody against angiotensin-converting enzyme (ACE) has been described which possesses very similar properties(Danilov et al. 1991 *Lab. Invest.* 64:118-124). Therapeutic enzymes such as catalase, superoxide dismutase and plasminogen activators conjugated with ACE antibody have been demonstrated to accumulate in the lungs after intravascular injection (Muzykantov et al. 1996 *Proc. Nat'l Acad. Sci. USA* 93:5213-5218; Muzykantov et al.

1997 *J. Pharm. Exp. Therap.* 279:1026-1034). In addition, an antibody against E-selectin has been described which binds to and delivers liposomes to the cytokine-activated endothelium in cell culture (Spragg et al. 1997 *Proc. Nat'l Acad. Sci. USA* 94:8795-8800). A PECAM antibody conjugated with streptavidin has also been recently described which provides an effective carrier for delivery of drugs to the endothelium (Muzykantov et al. 1998 *Am. J. Resp. Crit. Care Med.* 157:A203).

However, endothelial cells internalize antibodies against thrombomodulin (Muzykantov et al. 1997 *Circulation* 96:I43-44), ACE (Muzykantov et al. 1996 *Am. J. Physiol.* 270:L704-713), E-selectin (Spragg et al. 1997 *Proc. Nat'l Acad. Sci. USA* 94:8795-8800) and anti-PECAM/streptavidin complex (Muzykantov et al. 1998 *Am. J. Resp. Crit. Care Medicine* 157:A203). Thus, while these carriers provide intracellular delivery, a feature which may be useful for targeting of genes and some other therapeutic agents, anticoagulants or fibrinolytics must escape internalization and remain on the luminal surface in order to control blood components. Accordingly, these carrier antibodies are of limited use in the delivery of anticoagulants, fibrinolytics or other drugs wherein their therapeutic action is localized to the blood.

An ICAM-1 monoclonal antibody, mAb 1A29 has also been described which accumulates in rat lungs following i.v. injection. Conjugation of catalase to this ICAM-1 monoclonal antibody via a streptavidin-biotin crosslinker resulted in accumulation of the anti-ICAM-1 conjugated catalase in the lung and protection of the lung from damage by extracellular oxidants (Muzykantov et al. *Am. J. Resp. Crit. Care Medicine* 1997 155(4):p.A187). Radiolabeled mAb 1A29 has also been shown to accumulate in the vasculature challenged with pro-inflammatory agents TNF and endotoxin (Mulligan et al. 1993 *Am. J. Pathol.* 142:1739-1749). In addition, this

antibody has been shown to react with normal endothelial cells in the rat vasculature and that injection of TNF or endotoxin stimulates endothelial binding of mAb 1A29 (Panes et al. 1995 *Am. J. Physiol.* 269:H1955-1964). This antibody has also been shown to attenuate vascular injury induced by activated leukocytes via blocking of their adhesion to the endothelial cells.

It has now been found that monoclonal antibodies against the endothelial surface antigen ICAM-1 bind effectively to the endothelial cells without subsequent internalization. Conjugation of a drug to an non-internalizable antibody such as the ICAM-1 monoclonal antibody which binds to an antigen on the luminal surface of the pulmonary vasculature provides a useful means for targeted delivery and retention of the drug on the luminal surface, or blood compartment, of the pulmonary vasculature.

Summary of the Invention

An object of the present invention is to provide a method for targeting and prolonging association of drugs, the therapeutic action of which must be localized in the blood compartment of the pulmonary vasculature, to the luminal surface of either normal or inflammation-affected pulmonary vascular endothelium, which comprises utilization of a non-internalizable antibody which binds to an antigen on the luminal surface of the pulmonary vasculature, for example an anti-ICAM-1 antibody such as anti-ICAM-1 mAb 1A29, as an affinity carrier or a membrane anchor for the targeting and retention of the drugs on the luminal surface of the endothelium.

Another object of the present invention is to provide a method of administration of a drug, the therapeutic action of which must be localized in the blood compartment of the pulmonary vasculature which comprises either: I) conjugation of a selected drug with a non-internalizable antibody which

binds to an antigen on the luminal surface of the pulmonary vasculature, for example an anti-ICAM-1 antibody such as anti-ICAM-1 mAb 1A29, leading to formation of a "non-internalizable antibody/drug" complex and systemic administration of the said complex to an animal; or ii) step-wise systemic administration to an animal of a non-internalizable antibody which binds to an antigen on the luminal surface of the pulmonary vasculature followed by systemic administration of a drug chemically modified in the way that allows the drug to recognize and bind to the non-internalizable antibody bound to the luminal surface to avoid internalization.

Another object of the present invention is to provide a method for dissolution of fibrin clots or attenuation of the intravascular coagulation in the lung of an animal which comprises systemically administering to the animal a fibrinolytic or anticoagulant agent in combination with a non-internalizable monoclonal antibody which binds to an antigen on the luminal surface of the pulmonary vasculature.

Detailed Description of the Invention

ICAM-1 (InterCellular Adhesion Molecule-1) is a transmembrane protein anchored in the plasma membrane of several cell types, including endothelial cells. ICAM-1 is present on the surface of normal (non-stimulated) endothelium. Inflammatory agents cause elevation of ICAM-1 levels on the endothelial surface. Thus, inflammation-engaged endothelium possesses even more binding sites for ICAM-1 antibody than normal endothelium.

Monoclonal antibodies against ICAM-1 have been demonstrated to be useful as carriers of agents to the pulmonary endothelium. Accordingly, studies were performed to ascertain the usefulness of these anti-ICAM-1 antibodies in delivery of drugs such as fibrinolytics and anticoagulants. Anti-ICAM-1 mAb 1A29, a monoclonal antibody serving as an

example in the present invention which is a mouse IgG1 class monoclonal antibody reacting with rat ICAM-1, was used in these studies. This antibody is commercially available from a number of vendors including PharMingen (San Diego, CA), Endogen, Inc. (Boston, MA) and Serotec Ltd (United Kingdom). Since lack of internalization is an obligatory for the therapeutic action of fibrinolytics and anticoagulants, studies were performed to determine how endothelial cells in cell culture or in the lung blood vessels internalize radiolabeled mAb 1A29. These experiments demonstrated that unlike antibodies to other endothelial antigens, endothelial cells internalize anti-ICAM-1 extremely poorly.

For example, in cell culture, internalization of ^{125}I-mAb 1A29 did not exceed 5-10%. In contrast, ^{125}I-mAb against other endothelial antigens such as thrombomodulin and ACE displayed 60-80% internalization.

In addition, pulmonary uptake of anti-ICAM-1 is independent of the temperature thus indicating that this antibody is not internalized. Uptake of ^{125}I-mAb 1A29 in the isolated perfused rat lung was 18.7±3.2% at 37°C and 18.1±3.3% at 4°C. In contrast, pulmonary uptake of ^{125}I-mAb against ACE was twice as low at 4°C as compared to 37°C (Muzykantov et al. 1996 *Am. J. Physiol.* 270:L704-713).

Further, experiments conducted to evaluate whether mAb 1A29 associated with the endothelial cells disappears from the luminal surface in the lung showed that in sharp contrast to anti-ACE and other known carrier antibodies, anti-ICAM-1 is bound to the external surface of the pulmonary endothelial cells for a prolonged time and does not disappear from the lumen. In these experiments, rat lungs were perfused with biotinylated mAb 1A29 (b-mAb 1A29) and after elimination of non-bound antibody consequently perfused ^{125}I-streptavidin in the lungs. Pulmonary uptake of ^{125}I-streptavidin was at the same level when streptavidin was added to the perfusion either 5 or 60 minutes after elimination of b-mAb 1A29. In contrast,

it has been shown that consequent uptake of ^{125}I-streptavidin decreases dramatically within an hour after elimination of the biotinylated anti-ACE from the perfusate, thus indicating that b-anti-ACE disappears from the luminal surface.

Taken together, these results indicate that endothelial cells effectively bind anti-ICAM-1 antibodies such as mAb 1A29, yet do not internalize this carrier. Accordingly, drugs targeted to endothelial cells by anti-ICAM-1 will be exposed to the vascular lumen for a prolonged period of time and, therefore, will able to more effectively interact with plasma protein thus regulating coagulation and fibrinolysis.

Pulmonary uptake of fibrinolytics, namely, ^{125}I-tPA and ^{125}I-streptokinase conjugated with anti-ICAM-1 mAb 1A29, in the perfused rat lungs and after injection *in vivo* in rats was evaluated. As Table 1 shows, antibody-conjugated fibrinolytics, but not control IgG-conjugated enzymes accumulate in the rat lungs in both models, thus indicating that anti-ICAM-1 antibody indeed provides delivery of therapeutics to the luminal surface of the pulmonary vascular endothelium.

Table 1. Pulmonary uptake of radiolabeled therapeutic enzymes conjugated to either control IgG or to anti-ICAM-1 mAb 1A29.

	Carrier	Streptokinase	tPA
Perfused lung	IgG	1.3±0.7	1.6±0.4
Perfused lung	anti-ICAM	12.4±1.7	15.3±1.6
Lung, *in vivo*	IgG	ND	0.22±0.1
Lung, *in vivo*	anti-ICAM	ND	6.1±0.7

Data in Table 1 are presented as % of injected dose per gram of the lung tissue, M±SD, n=3. Radioactivity in the lung was determined 1 hour after start of the perfusion or after intravenous injection in intact anesthetized rats.

Further, subsequent perfusion of ^{125}I-tPA/streptavidin complex 60 minutes after accumulation of biotinylated mAb 1A29 in the lungs provided pulmonary uptake of 17.5±2.7% of ^{125}I-tPA. In a control experiment, in the absence of the first step of the targeting (i.e., without perfusion of biotinylated mAb 1A29) uptake of ^{125}I-tPA was equal to 0.7±0.2%, thus demonstrating the specificity of the targeting to b-anti-ICAM-1 attached to the pulmonary endothelium. Comparison of the result of step-wise targeting described above (17.5±2.7%) with that of direct targeting (15.3±1.6%, see Table 1) provides additional evidence that endothelial cells in the lung do not internalize mAb 1A29 as step-wise targeting would clearly be compromised by disappearance of b-mAb 1A29 from the lumen.

The functional activity of tPA targeted to the pulmonary endothelium via an anti-ICAM-1 monoclonal antibody was also evaluated. In these experiments, isolated rat lungs were perfused for 1 hour with 100 μg of mAb 1A29/tPA or IgG/tPA or with buffer. After elimination of non-bound material, lung tissue homogenates were prepared. Samples of lung homogenates were added to radiolabeled fibrin clot and incubated for 90 minutes at 37°C. Homogenate obtained from lungs perfused with conjugate-free buffer induced 6.5±1.0% fibrinolysis (background level). The homogenate obtained from the lungs perfused with IgG/tPA complex induced 9.2±2.5% fibrinolysis. This value is not significantly different from the background fibrinolysis level. In a sharp contrast, homogenate obtained from the lungs perfused with anti-ICAM-1/tPA complex induced 21.2±3.9 fibrinolysis. Thus, anti-ICAM-1-directed targeting of tPA to the luminal surface of the pulmonary endothelium markedly enhances fibrinolytic activity of the lung vasculature. Further, immunotargeting of tPA (or other plasminogen activators) will augment local fibrinolytic potential of endothelium in the focus of the pulmonary vasculature due to local generation of plasmin.

Accordingly, administration of a non-internalizable antibody such as anti-ICAM-1 antibody in combination with a selected drug, the therapeutic action of which must be localized in the blood compartment of the pulmonary vasculature, provides a useful means for targeting and prolonging association of the drug to the luminal surface of either normal or inflammation-affected pulmonary vascular endothelium.

By "non-internalizable antibody" it is meant an antibody which binds to an antigen on the luminal surface of the pulmonary vasculature such as the anti-ICAM-1 antibody, mAb 1A29, which is determined not to be internalized by cultured human endothelial cells as described in Example 2 and/or is shown to be temperature independent in pulmonary uptake experiments in isolated lung perfusions as described in Example 3. Non-internalizable antibodies other than the anti-ICAM-1 antibody described herein which are also useful in the instant invention can thus be identified routinely by those of skill in the art in accordance with teachings provided herein.

By "selected drug" in the present invention, it is meant to include any therapeutic agent, the therapeutic action of which must be localized in the blood compartment of the pulmonary vasculature. Examples include, but are not limited to fibrinolytics including plasminogen activators and anticoagulants.

By "prolonging association", it is meant that the drug when administered in combination with a non-internalizable antibody such as anti-ICAM-1 antibody undergoes slower inactivation and/or elimination from the bloodstream as compared to the same drug administered alone.

By "in combination" it is meant that a selected drug is administered either as a non-internalizable antibody/drug complex or in a stepwise manner wherein the non-internalizable antibody is administered first followed by administration of

the selected drug. Thus, in one embodiment, a selected drug can be conjugated to a non-internalizable antibody to form an non-internalizable antibody/drug complex by a number of different methods well known to those of skill in the art. For example, conjugation of anti-ICAM-1 to a selected drug such as a plasminogen activator may be performed using a homo-bifunctional cross-linking agent. Such cross-linking agents offer conjugation of two proteins via chemical modification of the same functional groups on both proteins. Since all proteins contain amino groups, this class of cross-linkers usually produces intermolecular complexes by cross-linking of their amino groups (Sakharov et al. 1988 *Thrombosis Res.* 49:481-488). Introduction of disulfide groups in two proteins by incubation with equimolar amounts of N-succinimidyl-3-(2-pyridildithio)propionate (SPDP) followed by reduction of the disulfide groups on one of the proteins also allows for conjugation of the two proteins (Cavallaro et al. 1993 *J. Biol. Chem.* 268:23186-23190). The hetero-bifunctional cross-linking agent, m-maleimidobenzoic acid N-hydroxysuccinimide ester can also be used for conjugating an SPDP-treated plasminogen activator with any protein including anti-ICAM-1 or, vice versa, SPDP-treated anti-ICAM-1 with a plasminogen activator. A selected drug can also be coupled with the antibody using a bi-functional antibody chimera possessing affinity for both the selected drug and the antibody. In a preferred embodiment, streptavidin-biotin cross-linking is used. In this embodiment, both the antibody and selected drug are modified with biotin ester which allows for further intermolecular conjugation of the biotinylated molecules by streptavidin. Streptavidin-mediated cross-linking of biotinylated proteins is a widely used biochemical method. Further, as demonstrated herein, the enzymatic activity of tPA is not reduced in the course of biotinylation, conjugation with streptavidin and with biotinylated anti-ICAM-1. In

addition, the ability of the antibody to specifically target the lung is not altered by this process.

In another embodiment, the selected drug is chemically modified to recognize and bind a non-internalizable antibody such as anti-ICAM-1 antibody associated with or bound to the luminal surface of the endothelium. In this embodiment, referred to herein as step-wise systemic administration, biotinylated non-internalizable antibody is systemically administered to the animal so that the antibody binds to a specific antigen on the luminal surface of the pulmonary vasculature. The selected chemically modified drug is then systemically administered to the animal so that the selected drug binds to the non-internalizable antibody associated with the luminal surface thereby avoiding internalization. For example, the plasminogen activator, tPA, has been chemically conjugated with streptavidin, a molecule recognizing a biotinylated anti-ICAM-1 antibody associated with endothelial surface. Thus tPA/streptavidin complex binds to endothelium-bound anti-ICAM antibody.

Administration of a selected drug in combination with a non-internalizable antibody is particularly useful in dissolution of fibrin clots or prevention of the intravascular coagulation in the lung. In this embodiment, it is preferred that the selected drug be a fibrinolytic agent, preferably a plasminogen activator, or an anticoagulant such as chemically modified heparin, hirudin or recombinant thrombomodulin.

By "systemic administration" it is meant to include intravenous, intraarterial injections and infusions, as well as local delivery via a vascular catheter into selected vascular bed (for example, pulmonary artery).

By "animal" it is meant to include mammals, most preferably humans.

The following nonlimiting examples are provided to further illustrate the present invention.

EXAMPLES

Example 1: Biotinylation, radiolabeling of proteins, and preparation of the conjugates

Biotin ester, 6-biotinylaminocaproic acid N-hydroxysuccinimide ester (BxNHS) was dissolved in 100% dimethylformamide to a final concentration of 10 mM or 1 mM. Control mouse IgG and anti-ICAM-1 mAb 1A29 were biotinylated at ten-fold molar excess of BxNHS. Eight μl of fresh 1 mM BxNHS were added to 100 μl of antibody solution (1 mg/ml in borate buffered saline, BBS, pH 8.1). After a 1 hour incubation on ice, an excess of non-reacted BxNHS was eliminated by overnight dialysis. Streptokinase and tPA were biotinylated by the same reagent at 10-fold molar excess of BxNHS, as described above. Biotinylated antibodies, b-streptokinase, b-tPA or streptavidin were radiolabeled with ^{125}Iodine using Iodogen-coated tubes according to the manufacturer's recommendations (Pierce). Incubation of 100 μg of a biotinylated protein and 100 μCi of Sodium ^{125}Iodide in a tube coated with 100 μg of Iodogen for 20 minutes on ice yields streptavidin with a specific radioactivity of approximately 500 cpm per ng. An excess of iodine was eliminated by dialysis. More than 95% of radiolabeled proteins were precipitable by TCA.

Tri-molecular heteropolymer complexes b-tPA/SA/b-IgG, b-tPA/SA/b-anti-ICAM-1 , b-streptokinase/SA/b-IgG and b-streptokinase/SA/b-anti-ICAM-1 were prepared by a two-step procedure. Specifically, at the first step, streptavidin (SA) and b-tPA were mixed at a molar ratio equal to 2, in order to form bi-molecular complexes b-tPA/SA. Accordingly, 10 μl of BBS containing 10 μg of radiolabeled b-tPA was mixed with 10 μl of BBS containing 20 μg of streptavidin and incubated for 1 hour on ice. The mixture was then divided into two 10 μl portions. To the first portion was added 15 μl of BBS containing 20 μg of biotinylated anti-ICAM-1. To the second portion was added 15 μl of BBS containing 20 μg of control

IgG. These mixtures were then incubated for two hours on ice, in order to form tri-molecular conjugates b-catalase/SA/b-anti-ICAM or b-catalase/SA/b-IgG. The same procedure has been utilized to generate tri-molecular complexes b-streptokinase/SA/b-IgG, b-streptokinase/SA/b-anti-ICAM.

Example 2: Interaction of radiolabeled antibodies with cultured human endothelial cells

Cultivated cells (HUVEC) were cultured in gelatin-coated plastic dishes ("Falcon") using Medium 199 with Earle's salts supplemented with 10% fetal calf serum, 200 μg/ml endothelial growth factor from human brain and 100 μg/ml heparin, 2 mM glutamine, 100 mU/ml penicillin and 100 μg/ml streptomycin. Cells were subcultivated from first to third passage by treatment with 0.05% trypsin/0.02% EDTA mixture.

To determine the internalization of antibodies by the endothelium, cells were incubated with 300 μl of culture medium containing 1 μg ^{125}I-anti-ICAM for 90 minutes at 37 °C. After washing to remove unbound radioactivity, cells were incubated with 50 mM glycine, 100 mM NaCl, pH 2.5 (15 minutes at room temperature) to release surface associated antibody. There was no detectable cell detachment after treatment with glycine buffer as determined by light microscopy. After collection of the glycine eluates, cells were detached by incubation with standard trypsin/EDTA solution. Surface associated radioactivity (i.e., radioactivity of the glycine eluates) and cell associated radioactivity (i.e., radioactivity of trypsin/EDTA extracts) were determined in a gamma counter. Percent of internalization was calculated as % = (total radioactivity - glycine eluted) x 100/total radioactivity.

SUBSTITUTE SHEET (RULE 26)

Example 3: Temperature dependence of pulmonary uptake of anti-ICAM-1

Sprague-Dawley male rats, weighing 170-200 grams, were anesthetized with sodium pentobarbital, 50 mg/kg, i.p., and prepared for isolated lung perfusion using recirculating perfusate. The trachea was cannulated and lungs were ventilated with a humidified gas mixture (Airco Inc., Philadelphia, PA) containing 5% CO_2 and 95% air. Ventilation was performed using a SAR-830 rodent ventilator (CWE Inc., Ardmore, PA) at 60 cycles/minute, 2 ml tidal volume, and 2 cm H_2O end-expiratory pressure. The thorax was then opened and a cannula was placed in the main pulmonary artery through the transected heart. The lungs were isolated from the thorax and initially perfused in a non-recirculating manner for a 5 minute equilibration period, in order to eliminate blood from the pulmonary vascular bed. Then lungs were transferred to the water-jacketed perfusion chamber maintained at 37°C or 6°C. Perfusion through the pulmonary artery was maintained by a peristaltic pump at a constant flow rate of 10 ml/minutes. The perfusate (45 ml per lung) was Krebs-Ringer buffer (KRB, pH 7.4), containing 10 mM glucose and 3% fatty acid-free BSA (KRB-BSA). Perfusate was filtered through a 0.4 μm filter prior to perfusion to eliminate particulates. To quantitate antibody binding, 1 μg of ^{125}I-labeled anti-ICAM-1 antibody 1A29 was added to the perfusate. Perfusate circulated for 60 minutes at either 37°C or 4°C. Then non-bound material was eliminated by 5 minutes non-recirculating perfusion of antibody-free KRB-BSA. Radioactivity in the lungs was measured in a gamma-counter and expressed as a percentage of perfused radioactivity per gram of lung tissue (% ID/g).

Example 4: Rat lung perfusions with biotinylated mAb 1A29

Perfusion of isolated rat lungs was performed as above. At the first step, 10 μg of non-labeled biotinylated anti-ICAM-1 antibody was added to the perfusate and circulated for

30 minutes at 37°C, to allow for antibody binding with the pulmonary endothelium. Thereafter, non-bound antibody was eliminated as above and perfusate was replaced with antibody-free KRB-BSA. Lungs were further perfused for either 5 minutes (to minimize antibody internalization) or 60 minutes at 37°C (to allow for antibody internalization). At the indicated time, 1 μg of ^{125}I-labeled streptavidin was perfused for 15 minutes with recirculating perfusion at 37°C followed by 5 minutes with non-recirculating perfusion with KRB-BSA to eliminate non-bound material. Radioactivity in the lungs was measured in a gamma-counter and expressed as a percentage of perfused radioactivity per gram of lung tissue (% ID/g).

Example 5: Pulmonary uptake of ^{125}I-tPA and ^{125}I-streptokinase conjugated with anti-ICAM-1 mAb 1A29 in perfused rat lungs

To study pulmonary uptake of radiolabeled preparations in blood-free buffer, 0.5 ml of saline containing 1 μg of radiolabeled b-streptokinase or b-tPA conjugated with anti-ICAM-1 antibody 1A29 was added to the perfusate and circulated in the isolated rat lungs for 1 hour at 37°C, as described above. Control lungs were perfused with complexes containing b-IgG instead of b-anti-ICAM-1. After a one hour perfusion, non-bound material was eliminated and lung-associated radioactivity was determined as above.

Example 6: Pulmonary uptake of ^{125}I-tPA and ^{125}I-streptokinase conjugated with anti-ICAM-1 mAb 1A29 after injection *in vivo* in rats

To study biodistribution of radiolabeled preparations in rats, an injection of 0.5 ml of saline containing 1 μg of radiolabeled b-streptokinase or b-tPA conjugated with anti-ICAM-1 antibody 1A29 was made into the tail vein under anesthesia. Control animals were injected with complexes containing b-IgG instead of b-anti-ICAM-1. Animals were

sacrificed by exsanguination 60 minutes after injection. Internal organs were washed with saline to remove blood and radioactivity in tissues was determined in a Rack-Gamma counter. The data were calculated as mean±standard error (M±SE). Statistical comparisons were made using one-way analysis of equal variance (ANOVA) followed by Student-Newman-Keuls Method. The level of statistical significance was taken as $p<0.05$.

Example 7: Functional activity of tPA in isolated rat lungs

To characterize functional activity of tPA conjugated with anti-ICAM antibody, 0.5 ml of saline containing 1 μg of b-tPA conjugated with anti-ICAM-1 antibody 1A29 was added to the perfusate and circulated in the isolated rat lungs for 1 hour at 37°C, as described above. Control lungs were perfused with complexes containing b-IgG instead of b-anti-ICAM-1. After a one hour perfusion, non-bound material was eliminated and lung homogenates were prepared. To test fibrinolytic activity of the homogenates, radiolabeled fibrin clot was prepared by addition of 50 μl of thrombin solution (1 μg/ml in saline) to a solution of radiolabeled human fibrinogen (3 mg/ml in KRB). Immediately after thrombin addition, aliquots of the solution (300 μl) were made and allowed to polymerize (60 minutes at room temperature). This procedure provides standard fibrin clots containing radiolabeled fibrin. Saline (1 ml) containing 50 ml of the homogenates prepared from lungs perfused with either b-tPA/SA/b-anti-ICAM or b-tPA/SA/b-IgG complexes (see above) was added to fibrin clots. After a 2 hour incubation at 37°C, radioactivity in the supernatants was determined. Percent of fibrinolysis was expressed as percent of the radioactivity in the supernatants (i.e., radioactivity of the products of fibrin degradation) to the total radioactivity of fibrin clots.

What is Claimed is:

1. A method for targeting and prolonging association of a selected drug to the luminal surface of pulmonary vascular endothelium of an animal comprising administering to an animal a selected drug in combination with a non-internalizable antibody which binds to an antigen on the luminal surface of the pulmonary vascular endothelium.

2. The method of claim 1 wherein the non-internalizable antibody is an anti-ICAM-1 antibody.

3. The method of claim 1 wherein the selected drug is conjugated with the non-internalizable antibody.

4. The method of claim 1 wherein the non-internalizable antibody is administered to the animal followed by administration of the selected drug wherein the selected drug has been chemically modified to recognize the non-internalizable antibody associated with luminal surface of the pulmonary vasculature.

5. A method for dissolution of fibrin clots or prevention of the intravascular coagulation in the pulmonary vasculature of an animal comprising administering to the animal a fibrinolytic or anticoagulant in combination with a non-internalizable antibody which binds to an antigen on the luminal surface of the pulmonary vascular endothelium.

6. The method of claim 5 wherein the non-internalizable antibody is an anti-ICAM-1 antibody.

7. The method of claim 5 wherein the fibrinolytic or anticoagulant is conjugated with the non-internalizable antibody.

8. The method of claim 5 wherein the non-internalizable antibody is administered to the animal followed by administration of the fibrinolytic or anticoagulant wherein the fibrinolytic or anticoagulant has been chemically modified to recognize the non-internalizable antibody associated with luminal surface of the pulmonary vasculature.

Docket No.
PENN-0749

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Targeting and Prolonging Association of Drugs to the Luminal Surface of the Pulmonary Vascular Endothelial Cells

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on **August 2, 1999** as United States Application No. or PCT International Application Number **PCT/US99/17386**

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)
60/095,240	August 4, 1998

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Send Correspondence to: **Jane Massey Licata or Kathleen A. Tyrrell**
Licata & Tyrrell P.C.
66 E. Main Street
Marlton, New Jersey 08053

Direct Telephone Calls to: *(name and telephone number)*
Jane Massey Licata or Kathleen A. Tyrrell - (856) 810-1515

1-00

Full name of sole or first inventor
Vladimir R. Muzykantov

Sole or first inventor's signature — Date 6/28/01

Residence
~~Philadelphia, Pennsylvania~~ WARWICK, PENNSYLVANIA PA. VRM 6.28.01

Citizenship
~~US~~ RUSSIAN VRM 6.28.01

Post Office Address
~~110 Brookshire Place~~ 101 WHITE PINE COURT VRM 6.28.01

~~Philadelphia, Pennsylvania 19116~~ WARWICK, PENNSYLVANIA 18974 VRM 6.28.01

2-00

Full name of second inventor, if any
Juan Carlos Murciano

Second inventor's signature — Date 6/28/01

Residence
Sevilla, Spain ESX

Citizenship
Spain

Post Office Address ANTIOQUIA JCM 6/28/01
Building 4, #6B Avenida ~~Antiohia~~ 1

Sevilla, Spain E-41007

3-00

Full name of third inventor, if any Neil D. Granger	D. Neil Granger
Third inventor's signature	[signature] Date 6/28/01
Residence ~~[illegible]~~	Shreveport, Louisiana dng 6/28/01
Citizenship US	US
Post Office Address ~~[illegible]~~	1501 Kings Hwy dng 6/28/01
~~[illegible]~~	Shreveport, LA 71130-3932

dng 6/28/01

Full name of fourth inventor, if any	
Fourth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

Full name of fifth inventor, if any	
Fifth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

Full name of sixth inventor, if any	
Sixth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

10082503.062801